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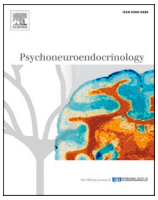

# Oxytocin system gene methylation is associated with empathic responses towards children

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ABSTRACT

Empathy is an essential component of sensitive caregiving behavior, which in turn is an important predictor of children's healthy social-emotional development. The oxytocin (OXT) system plays a key role in promoting sensitive parenting and empathy. In this study, we investigated how OXT system gene methylation was associated with empathic processes in nulliparous women (*M* age = 23.60, *SD* =0.44)—measuring both physiological facial muscle responses and ratings of compassion and positive affect to affective images depicting children. Linear mixed effects analyses demonstrated that lower methylation levels in the *OXT* and *OXTR* genes were related to enhanced empathic responses. The effect of OXT system gene methylation on empathic processes was partly qualified by an interaction with individual variations in women's care motivation. Our findings provide experimental evidence for an association between the methylation of OXT system genes and empathy.

## 1. Introduction

Empathy involves the understanding and sharing of the affective states of others (De Waal and Preston, 2017; Decety and Jackson, 2004). As such, empathy is a crucial component of sensitive caregiving, which refers to caregivers' adequate and prompt responses to the signals and needs of a child (Ainsworth et al., 1978; Bowlby, 1988). Many studies have demonstrated that empathy and sensitive parenting are both influenced by the neuropeptide oxytocin (OXT) (Bos, 2016; Feldman et al., 2015; Feldman and Bakermans-Kranenburg, 2017). For example, previous studies found that intranasally administered OXT and higher peripheral OXT concentrations increased responsiveness to infant cues (see, e.g., Riem et al., 2011; Strathearn et al., 2009). OXT administration furthermore improved the recognition of emotional facial expressions (Leppanen et al., 2017), and promoted empathic responses (Bartz et al., 2010).

A central question in OXT research has been how the genetic variability of OXT system genes can influence social functioning in humans. However, previous studies that tested the effect of allelic variations of OXT system genes on social behavior provided mixed and inconclusive findings (Bakermans-Kranenburg and van IJzendoorn, 2014; Feldman et al., 2015). This may be due to very small effect sizes of individual allelic variations on complex traits, which thus require large sample sizes to be detected (Chabris et al., 2013). A possible further explanation for this unresolved question is that regulation of gene activity by epigenetic mechanisms also plays a role in these behavioral outcomes, and only recently have studies started to take these mechanisms into account. Epigenetic mechanisms are chemical adaptations of chromosomal regions of DNA that alter gene activity without changing the underlying gene sequence (Bird, 2007). DNA methylation is the most stable and well-studied epigenetic process and when methylation occurs in transcription sites in the promotor region of a gene, this can lead to reduced gene expression (Szyf and Bick, 2013).

Limited studies have examined a direct relation between social behavior and methylation of OXT system genes in humans, with a focus on the *OXT* and OXT-receptor (*OXTR*) gene. The *OXT* gene encodes a precursor protein that is synthesized to produce OXT (Haas et al., 2016). The effects of OXT on brain functioning and behavior are dependent on the expression of its receptor, which is encoded by the *OXTR* gene (Puglia et al., 2015). Thus, *OXTR* methylation may indirectly reduce

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OXT functioning by negatively affecting *OXTR* expression levels (Kraaijenvanger et al., 2019). Lower methylation levels of the *OXT* and *OXTR* gene have been related to improved emotion recognition (Haas et al., 2016; Rubin et al., 2016), enhanced emotion regulation (Puglia et al., 2015), and enhanced attention towards social information (Puglia et al., 2018, but for an interaction with childhood trauma see Parianen Lesemann et al., 2020). As such, lower levels of *OXT* or *OXTR* gene methylation are suggested to enhance social sensitivity (Kraaijenvanger et al., 2019). This hypothesis is substantiated by studies reporting higher *OXTR* gene methylation levels, compared to controls, in individuals affected by psychopathologies characterized by impairments in social functioning, such as autism spectrum disorders, depressive disorders, and externalizing disorders (Kraaijenvanger et al., 2019).

The main goal of this study was to experimentally test if methylation of OXT system genes is associated with empathic processes in response to children, since such evidence is currently lacking. We therefore measured empathic responses towards images depicting children in positive social interactions and socially distressing scenes using facial electromyography (EMG), as well as behavioral ratings of compassion and affect. Facial EMG is often used to examine empathic processes in response to affective stimuli that generally elicit rapid, automatic changes in facial muscle activity and are associated with changes in affective states (Kraaijenvanger et al., 2017). Typically, negative stimuli enhance corrugator supercilli (COR) activity, involved in frowning expressions, which is related to a negative mood state. On the other hand, positive stimuli elicit zygomaticus major (ZYG) activity, involved in smiling expressions, which is related to a positive mood state (Lang et al., 1993). Generally, highly empathic individuals are more facially reactive to emotional stimuli (Dimberg et al., 2011; Van der Graaff et al., 2016). Furthermore, limited evidence suggests that OXT administration increases facial reactivity to emotional faces (Korb et al., 2016, but see Trilla et al., 2020).

Interestingly, effects of OXT system functioning on empathic processes may be constrained by individual dispositional factors. For example, studies demonstrated that OXT administration can differentially affect social cognitive processes or prosocial behavior, dependent on individuals' attachment security and gender (Bartz et al., 2011). In the context of caregiving, individual variations in caregiving motivation may explain differences in the degree to which the OXT system facilitates responsiveness to children (Bos et al., 2018). Previous studies indeed demonstrated that care motivation was positively associated with neural and behavioral reward responses to infant and child cues (Buckels et al., 2015; Endendijk et al., 2018; Spencer et al., 2018), and one study further demonstrated that this association interacted with the effect of OXT administration (Bos et al., 2018). Since positive effects of OXT administration on empathic processes were previously shown to be most pronounced in less socially competent individuals (Bartz et al., 2010), we therefore exploratively tested if effects of OXT system gene methylation on empathic processes were most pronounced in individuals with low intrinsic motivation for caregiving.

To summarize, in the current study we investigated how individual variations in *OXT* and *OXTR* gene methylation influenced empathic processes in response to affective images depicting children by measuring facial muscle responses and ratings of compassion and affect. Based on our literature review, we expected that lower levels of methylation in the *OXT* and *OXTR* genes were related to enhanced empathic processes. Furthermore, we exploratively tested if an interaction with individual variations in care motivation would further qualify this relation.

## 2. Methods

### 2.1. Participants

Participants were included from the RADAR (Research on Adolescent Development and Relationships) Young cohort (Branje and Meeus, 2018). Because our study was part of a series of studies on caregiving behavior prior to motherhood, only female nulliparous participants were approached for participation. They were screened on the following exclusion criteria: 1) pregnancy or motherhood, 2) history of endocrinal, neurological, or psychiatric conditions, and 3) use or previous use of medication that influences endocrinal, neurological, or psychological functioning. An initial sample size of 84 participants was aimed for, based on a power calculation for a multiple regression with four independent predictors, a medium effect size, .80 power, and .05 alpha-level (Cohen, 1992). Of all ($n$ = 154) young adult females contacted for screening, eighty-one eventually participated in the study ($M$ age = 23.60, $SD$ = 0.44, range = 22.30–24.76 years). Reasons for not being included in the study were: refusal to participate in the lab study ($n$ = 44), meeting exclusion criteria ($n$ = 18), or unreachable via telephone or email ($n$ = 11). The study was conducted in accordance with the latest version of the Declaration of Helsinki and was approved by the medical ethical committee of the UMC Utrecht (NL57474.041.16, METC 16/244). Upon arrival in the lab, all participants were informed about task procedures and gave written informed consent prior to study participation. As part of a larger series of studies on caregiving behavior prior to motherhood, during the lab visit participants provided two saliva samples for DNA methylation and steroid hormone assessment. Additionally, they completed five experimental computer tasks and a series of questionnaires. The measures that were part of the current study are described below.

### 2.2. . Affective stimuli and experimental task

Affective stimuli were thirty black and white images depicting children in a positive, negative, or neutral naturalistic context. Positive images depicted children in positive social interactions (e.g., a child reading a book with a parent). Negative images depicted children in socially distressing scenes (e.g., a begging street child). Neutral images depicted children in neutral scenes (e.g., a child playing on a computer with a neutral facial expression). Neutral images were included to allow us to control for baseline activity of facial muscles. The dimension of all images was 640 × 480 pixels and mean luminance was controlled for across conditions. During the experimental task, stimuli were presented in a randomized order at the center of the screen for a duration of 2 s and were preceded by a 1 s fixation cross. After each stimulus presentation, participants were required to report how much compassion they felt for the child in the picture (1 'not at all' to 9 'a lot') and how positive they felt in response to the picture (1 'not at all' to 9 'a lot'). There was no time limitation on participant's responses and responses were followed by a blank screen with a duration of 1 s. Intertrial durations varied according to participants' reaction time.

Images were selected out of a collection of 120 photographs obtained after an internet search. Compassion and positive affect in response to the images were reported by 24 independent female participants ($M$ age = 37.46, $SD$ = 15.03). Ten images per condition were selected with the most consistent reports on compassion and positive affect. The resulting conditions differed significantly on compassion, $F(2, 46) = 450.70$, $p < .001$, $\eta^2 = .95$, and positive affect, $F(2, 46) = 318.98$, $p < .001$, $\eta^2 = .93$. To test the feasibility of the paradigm using facial EMG, a pilot study was conducted with forty-one female participants ($M$ age = 21.54, $SD$ = 2.15). As expected, corrugator supercilii (COR) activity was significantly greater in response to negative images compared to positive images, $t(570.71) = 5.21$, $p < .001$, and zygomaticus major (ZYG) activity was significantly greater in response to positive images compared to negative images $t(612) = 4.90$, $p < .001$.

### 2.3. Electromyography (EMG) data collection and pre-processing

EMG activity was recorded during the experimental task from bipolar electrodes placed over the left ZYG to measure smiling responses, and over the left COR to measure frowning responses (Fridlund and

Cacioppo, 1986). The ground consisted of the active common mode sense (CMS) and passive driven right leg (DRL) electrodes. EMG activity was recorded at a sampling rate of 2048 Hz using a Biosemi ActiveTwo amplifier and stored for offline analysis. Data reduction was performed using Brain Vision Analyser 2. Raw EMG data was 30–500 Hz band pass filtered with a rolloff of 24 db and a notch filter of 50 Hz. For each trial, data was segmented into −1000 – 2000 ms epochs time-locked to stimulus onset. Signals were rectified and then baseline correction was applied by subtracting the averaged EMG activity 1000 ms pre-stimulus onset period from the post-stimulus onset values. Trials in which average post-stimulus onset EMG activity was ± 3 *SD* from the mean activity within subjects were rejected as artifacts (3.99% of total trials, no difference between conditions, *ts* < .19, *ps* > .07. EMG signals were averaged into 250 ms intervals per condition, resulting in 3 conditions with 8 post-stimulus onset time bins per participant. To control for baseline activity of facial muscles in response to neutral images of children, contrasts were calculated to represent the negative and positive condition by subtracting average EMG activity during the neutral condition from the negative and positive condition, respectively, for each timepoint. Finally, to prevent that extreme values disproportionally affect the analysis and activity curves over time, we winsorized EMG data to 95% confidence intervals within each timepoint before entering into statistical analyses.

### 2.4. Self-report measures

Participants filled out the *Nurturance* and *Protection* subscales of the Parental Care and Tenderness (PCAT) questionnaire to assess individuals' intrinsic motivation for caregiving (Buckels et al., 2015; Hofer et al., 2018). For this questionnaire, participants were first asked to rate how much they agree with a number of statements (example item nurturance subscale: "When I see infants, I want to hold them."; example item protection subscale: "I would hurt anyone who was a threat to a child."). Items were answered on a 5-point scale from 1 (*strongly disagree)* to 5 (*strongly agree)*. Next, participants were asked to rate how much tenderness they would feel in certain hypothetical situations and these items were only part of the nurturance subscale (example item: "You make a baby laugh over and over again by making silly faces."). Items were answered on a 5-point scale from 1 (*no tenderness at all)* to 5 (*a lot of tenderness)*. The nurturance subscale contained 6 items and the protection subscale contained 4 items. Nurturance and protection scores were computed as the mean response to the subscale items. In the current study, participants' mean nurturance score was 3.64 (*SD* = 0.66, Range = 1.50–4.83) and the mean protection score was 3.33 (*SD* = 0.77, Range = 1.75–5.00).

Additionally, to test whether facial muscle activity and ratings of compassion and positive affect in response to the affective images of children were related to empathy, participants filled out the *Empathic Concern* subscale of the Dutch version of the Interpersonal Reactivity Index (IRI; De Corte et al., 2007). This subscale contains 7 items and measures feelings of sympathy and concern for others (Davis, 1983). Items were answered on a 5-point scale from "*does not describe me well"* to "*describes me well"*. In the current study, the mean empathic concern score was 21.45 (*SD* = 3.62, Range = 12–29). Analyses included in Supplemental data demonstrated that participants with higher *Empathic Concern* scores had greater facial muscle responses and higher ratings of compassion and affect in response to the affective images of children. One participant did not complete the IRI.

### 2.5. DNA methylation collection and assessment

Saliva samples (2.0 mL) were collected after task execution using the Oragene•DNA (OG-500) Kit (DNA Genotek Inc., Ottowa, CA) and stored at room temperature until further DNA methylation analyses. Extracted DNA was submitted to bisulfite treatment before undergoing polymerase chain reaction (PCR) and Methylation Sensitive High-Resolution Melting (MS-HRM) analyses (Wojdacz and Dobrovic, 2007).

Based on the study by Haas et al. (2016), the primer set for the *OXT* gene covered the target sequence GRCh38/hg38, chr20: 3071297-3071697 (containing 17 CpG sites). The primer set for the *OXTR* gene was the same as used in the study by Bell and colleagues (2015), which was designed to cover target sequence GRCh38/hg38, chr3: 8769043-8769159 (containing 5 CpG sites, including CpG site -934 (hg38, 3:8769121)). Specifics of the *OXT* and *OXTR* primer sets are reported in the Supplemental material (see S1). Bisulfite PCR amplification of the target sequences was conducted in duplo and this was followed by HRM analyses to record the melting profile of the samples. The area under the curve was used to represent the methylation levels, which was the average measure of two replicates. The area under the curve provides an estimation of methylation levels across all CpG sites in the target sequences and does not provide methylation values on a single CpG site resolution. Average methylation level for the *OXT* gene was 214.35 (*SD* = 12.47, Range = 193.42–264.32). Average methylation level for the *OXTR* gene was 143.41 (*SD* = 6.19, Range = 132.58–166.32). The average across replicate CV was 3.67% for the *OXT* gene and 1.68% for the *OXTR* gene. Further details of the methylation assessment have been reported elsewhere (see supplementary material in Parianen Lesemann et al., 2020).

### 2.6. Statistical analyses

First, task effects were examined with linear mixed-effects analyses using the lmer and lm4 packages (Bates et al., 2015; Kuznetsova et al., 2017) in "R" Version 3.5.2 (R Core Team, 2018), with checkpoint function set to January 1st, 2019 to ensure reproducibility. We included the maximal random effects structure justified by our study design (Barr et al., 2013). P-values were obtained by significant omnibus F tests, using type III ANOVAs via Satterthwaite's degrees of freedom method (Kuznetsova et al., 2014). Continuous variables (including time) were standardized before they were entered into the models. Post-hoc contrast analyses were conducted by comparing least square means and trends using the lsmeans package (Lenth, 2016). Effects of affective condition were examined by including EMG data (for the COR and ZYG) and ratings (of compassion and positive affect) into separate models with affective condition (negative vs. positive) as fixed effect. For EMG data analyses, time was added as a fixed factor in interaction with affective condition. As random effects, we included intercepts for participants, as well as by-participant slopes for each fixed factor. This resulted in the following base model: EMG data / Ratings ~ Affective condition (* Time) + (1 + Affective condition (+ Time) | Participant).

Next, we tested the effects of *OXT* and *OXTR* methylation on our task outcomes by entering methylation levels into separate models in interaction with the fixed effects of the base model. This resulted in the following methylation model: EMG data / Ratings ~ *OXT/OXTR* methylation level * Affective condition (* Time) + (1 + Affective condition (+ Time) | Participant). Lower order interaction and main effects that confirmed task effects were omitted from the result sections for conciseness.

Finally, when *OXT* and/or *OXTR* interaction effects on task outcomes were significant, we examined if this effect was moderated by self-reported nurturance tendencies by entering nurturance scores into separate models in interaction with the fixed effects of the methylation model. Analyses revealed that only the nurturance subscale significantly interacted with OXT system gene methylation levels, therefore these are reported in the results section. Analyses with the protection subscale are reported in the Supplemental material. This resulted in the following model: EMG data / Ratings ~ Nurturance * *OXT/OXTR* methylation level * Affective condition (* Time) + (1 + Affective condition (+ Time) | Participant). Lower order interaction and main effects that confirmed task and methylation effects were omitted from the result sections for conciseness. Correlations between methylation levels and self-report measures were examined using two-tailed Spearman's correlation

analyses and results can be found in the supplementary material (see S2).

## 3. Results

### 3.1. Task effects

#### 3.1.1. EMG data analyses

*3.1.1.1. COR activity.* There was a significant interaction effect between affective condition and time on COR activity, $F(1|1052) = 420.53$, $p < .001$ There was also a main effect of affective condition, $F(1|80) = 84.40$, $p < .001$, yet no main effect of time, $p = .51$. Post-hoc tests revealed that over time COR activity was significantly greater in response to negative images compared to positive images, $t(1052) = 20.51$, $p < .001$ (see Fig. 1A).

*3.1.1.2. ZYG activity.* There was a significant interaction effect between affective condition and time on ZYG activity, $F(1|1052) = 182.06$, $p < .001$. There was also a main effect of affective condition, $F(1|80) = 24.12$, $p < .001$, and time, $F(1|80) = 18.26$, $p < .001$. Post-hoc tests revealed that over time ZYG activity was significantly greater in response to positive images compared to negative images, $t(1052) = 13.49$, $p < .001$ (see Fig. 1B).

#### 3.1.2. Behavioral rating analyses

*3.1.2.1. Reported compassion.* There was a significant effect of affective condition on reported compassion, $F(1|80) = 2762.00$, $p < .001$. Post-hoc test revealed that reported compassion was significantly higher for negative images compared to positive images, $t(80) = 52.55$, $p < .001$ (see Fig. 2A).

*3.1.2.2. Reported positive affect.* The model revealed a significant effect of affective condition on reported positive affect, $F(1|160) = 5661.50$, $p < .001$. Post-hoc test revealed that reported positive affect was significantly higher for positive images compared to negative images, $t(160) = 75.24$, $p < .001$ (see Fig. 2B).

### 3.2. Effect of OXT gene methylation

#### 3.2.1. EMG data analyses

*3.2.1.1. COR activity.* There was a significant three-way interaction between affective condition, time, and *OXT* methylation levels, $F(1|1051) = 18.97$, $p < .001$. Lower order effects of affective condition and the interaction between affective condition and time remained significant, $p$'s $< .001$. Post-hoc tests revealed that for both low (1 *SD* below the mean) and high (1 *SD* above the mean) *OXT* methylation levels, COR activity over time was significantly greater in response to negative images compared to positive images, $-1$ *SD:* $t(1051) = 17.70$, $p < .001$; $+1$ *SD*: $t(1051) = 11.54$, $p < .001$. Moreover, low *OXT* methylation levels were associated with a significantly greater differentiation in response to the positive and negative pictures compared to high *OXT* methylation levels, $t(1051) = 4.36$, $p < .001$ (see Fig. 3A).

*3.2.1.2. ZYG activity.* There was a significant three-way interaction between affective condition, time, and *OXT* methylation levels, $F(1|1051) = 10.77$, $p = .001$. Lower order effects of affective condition, time, and the interaction between affective condition and time remained significant, $p$'s $< .001$. Post-hoc tests revealed that for both low (1 *SD* below the mean) and high (1 *SD* above the mean) *OXT* methylation levels, ZYG activity over time was significantly greater in response to positive images compared to negative images, $-1$ *SD*: $t(1051) = 11.90$, $p < .001$; $+1$ *SD*: $t(1051) = 7.26$, $p < .001$. Moreover, low *OXT* methylation levels were associated with a significantly greater differentiation in response to the positive and negative pictures compared to high *OXT* methylation levels, $t(1051) = 3.28$, $p = .001$ (see Fig. 3B).

#### 3.2.2. Behavioral rating analyses

*3.2.2.1. Reported compassion.* There was no significant interaction between affective condition and *OXT* methylation levels, $p = .318$. The lower order effect of affective condition remained significant, $p < .001$.

*3.2.2.2. Reported positive affect.* There was a significant interaction between affective condition and *OXT* methylation levels, $F(1|158) = 4.31$, $p = .040$. The lower order effect of affective condition remained significant, $p < .001$. Post-hoc tests revealed that for both low (1 *SD* below the mean) and high (1 *SD* above the mean) *OXT* methylation levels, reports of positive affect were significantly greater for positive compared to negative images, $-1$ *SD*: $t(79) = 54.97$, $p < .001$; $+1$ *SD*: $t(79) = 52.03$, $p < .001$. Moreover, low *OXT* methylation levels were associated with a significantly greater differentiation in reports of positive affect in response to the positive and negative pictures compared to high *OXT* methylation levels, $t(79) = 2.08$, $p = .041$ (see Fig. 3C).

### 3.3. Effect of OXTR gene methylation

#### 3.3.1. EMG data analyses

*3.3.1.1. COR activity.* There was a significant three-way interaction

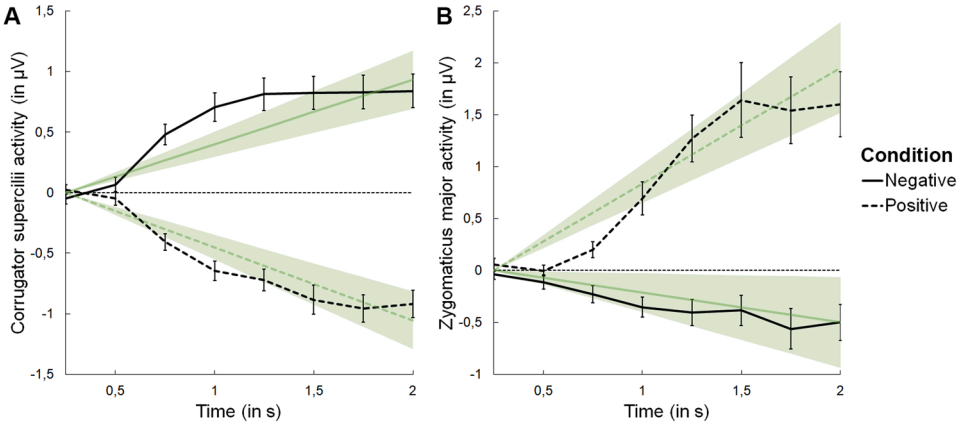


**Fig. 1.** EMG activity over time in response to negative and positive images for the COR (A) and ZYG (B) with error bars representing standard errors. Trend estimates are plotted with 95% CI bands.

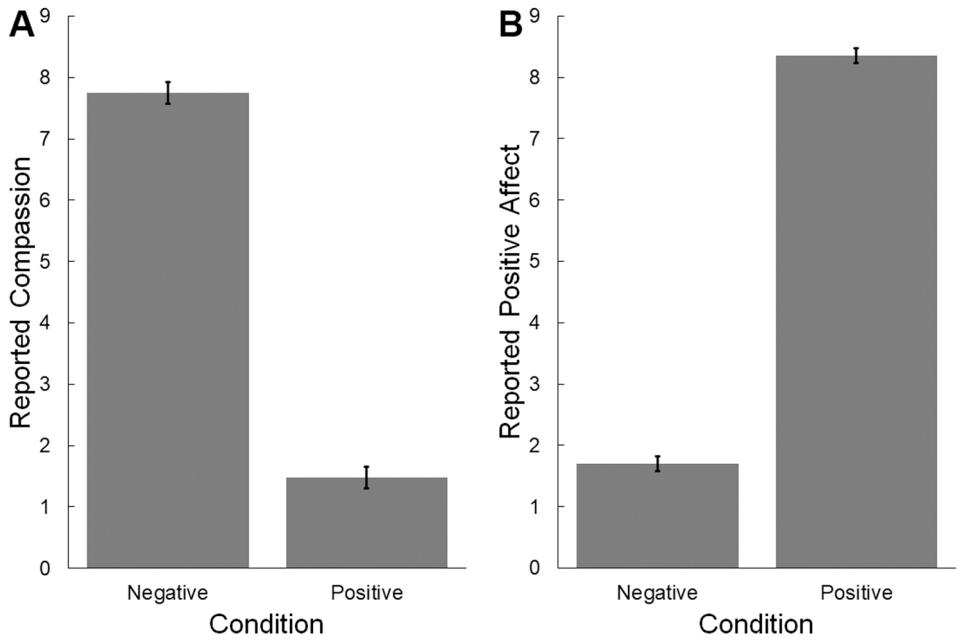


**Fig. 2.** Behavioral ratings of compassion (A) and positive affect (B) in response to negative and positive images. Error bars represent 95% CI.

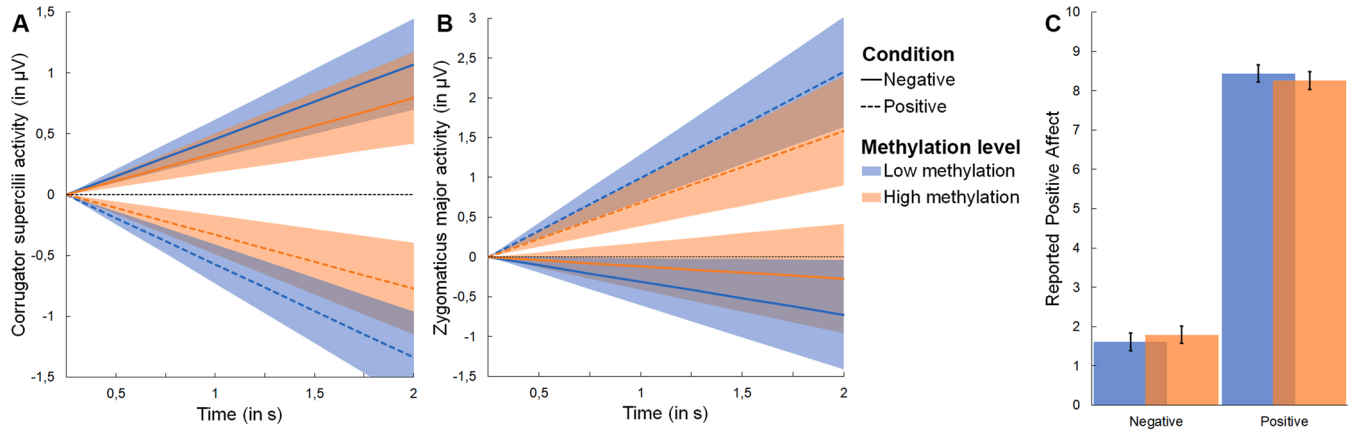


**Fig. 3.** Effect estimates for COR activity (A), ZYG activity (B), and reported positive affect (C) in response to negative and positive images for low (1 SD below the mean) and high (1 SD above the mean) *OXT* methylation levels with 95% CI bands and error bars.

between affective condition, time, and *OXTR* methylation levels, $F(1|1051) = 9.64$, $p = .002$. Lower order effects of affective condition and the interaction between affective condition and time remained significant, $p$'s $< .001$. Post-hoc tests revealed that for both low (1 *SD* below the mean) and high (1 *SD* above the mean) *OXTR* methylation levels, COR activity was significantly greater in response to negative images compared to positive images, $-1$ *SD*: $t(1051) = 16.75$, $p < .001$; $+1$ *SD*: $t(1051) = 12.36$, $p < .001$. Moreover, low *OXTR* methylation levels were associated with a significantly greater differentiation in response to the positive and negative pictures compared to high *OXTR* methylation levels, $t(1051) = 3.11$, $p = .002$ (see Fig. 4).

*3.3.1.2. ZYG activity.* There was no interaction effect with *OXTR* methylation levels on ZYG activity, $p = .491$. Lower order effects of affective condition, time, and the interaction between affective condition and time remained significant, $p$'s $< .001$.

### 3.3.2. Behavioral rating analyses

*3.3.2.1. Reported compassion.* There was no significant interaction effect of affective condition with *OXTR* methylation levels, $p = .170$. The lower order effect of affective condition remained significant, $p < .001$

*3.3.2.2. Reported positive affect.* There was no significant interaction effect of affective condition with *OXTR* methylation levels, $p = .914$. The lower order effect of affective condition remained significant, $p < .001$.

## 3.4. Interaction between nurturance tendencies and OXT methylation levels

### 3.4.1. EMG analyses

*3.4.1.1. COR activity.* The effect of *OXT* methylation on COR activity in response to the affective images was not further qualified by an interaction with nurturance tendencies, $p = .155$. Lower order effects of

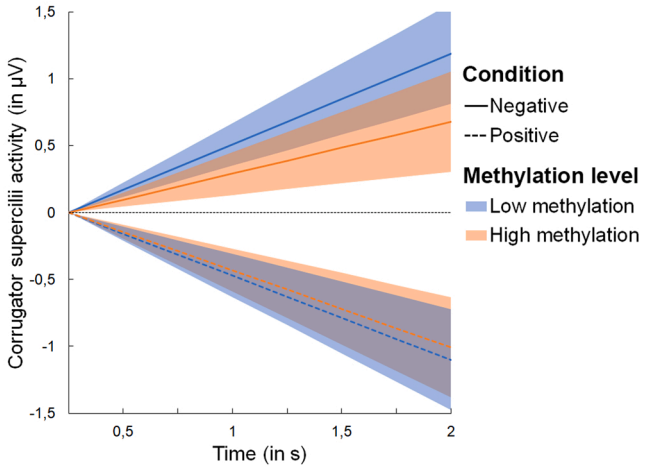


**Fig. 4.** Effect estimates for COR activity in response to negative and positive images for low (1 SD below the mean) and high (1 SD above the mean) *OXTR* methylation levels with 95% CI bands.

affective condition, the interaction between affective condition and time, and the interaction between affective condition, time, and *OXT* methylation remained significant, $p$'s $< .001$. There was a three-way interaction effect between affective condition, time, and nurturance tendencies on COR activity, $F(1|1049) = 7.66$, $p = .006$. Post-hoc tests revealed that for low (1 *SD* below the mean) and high (1 *SD* above the mean) nurturance tendencies, COR activity over time was significantly greater in response to negative images compared to positive images, $-1$ *SD:* $t(1049) = 12.44$, $p < .001$; $+1$ *SD*: $t(1049) = 16.63$, $p < .001$. Moreover, high nurturance tendencies were associated with a significantly greater differentiation in response to the positive and negative pictures compared to low nurturance tendencies, $t(1049) = 2.77$, $p = .006$.

*3.4.1.2. ZYG activity.* There was a significant four-way interaction between affective condition, time, *OXT* methylation, and nurturance tendencies, $F(1|1049) = 9.87$, $p = .002$. Lower order effects of affective condition, the interaction between affective condition and time, and the interaction between affective condition, time, and *OXT* methylation remained significant, $p$'s $< .01$. Post-hoc tests revealed that with *high* nurturance tendencies, ZYG activity was significantly greater in response to positive images compared to negative images for both low and high *OXT* methylation levels, $-1$ *SD:* $t(1049) = 7.30$, $p < .001$; $+1$ *SD*: $t(1049) = 7.74$, $p < .001$. With *low* nurturance tendencies ZYG activity was also significantly greater in response to positive images compared to negative images for both low *OXT* methylation levels and high *OXT* methylation levels, $-1$ *SD:* $t(1049) = 8.52$, $p < .001$; $+1$ *SD*: $t(1049) = 3.29$, $p = .023$. Moreover, with *low* nurturance tendencies, low *OXT* methylation levels were associated with a significantly greater differentiation in ZYG activity in response to the positive and negative pictures compared to high *OXT* methylation levels, $t(1049) = 4.46$, $p < .001$. With *high* nurturance tendencies, *OXT* methylation levels were not significantly associated with differences in ZYG activity in response to the affective images, $p = .987$. This suggests that nurturance tendencies buffered the effects of *OXT* methylation on ZYG activity in a way that high nurturance tendencies were associated with diminished effects of *OXT* methylation on ZYG activity compared to low nurturance tendencies. On the other hand, for low nurturance tendency scores, low

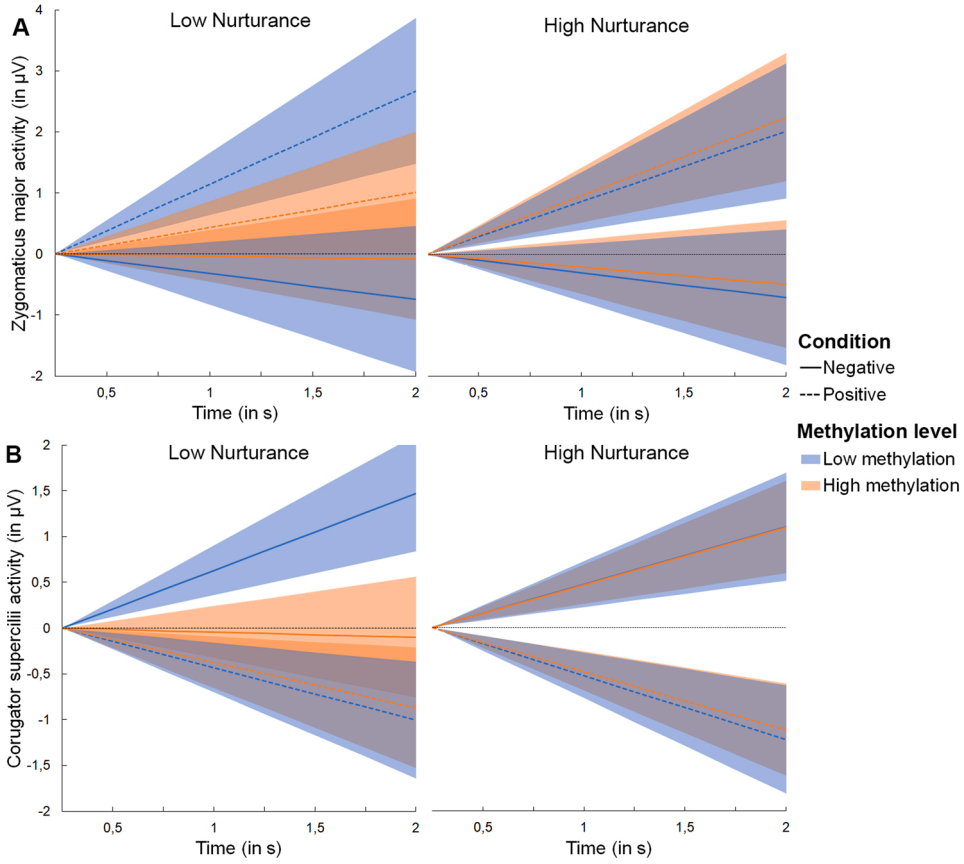


**Fig. 5.** Effect estimates for ZYG (A) and COR (B) activity over time in response to negative and positive affective images for low (1 SD below the mean) and high (1 SD above the mean) nurturance tendencies with 95% CI bands. ZYG activity is displayed for low (1 SD below the mean) and high (1 SD above the mean) *OXT* methylation levels. COR activity is displayed for low (1 SD below the mean) and high (1 SD above the mean) *OXTR* methylation levels.

*OXT* methylation was associated with more pronounced ZYG activity compared to high *OXT* methylation (see Fig. 5A).

#### 3.4.2. Behavioral rating analyses

*3.4.2.1. Reported compassion.* There was no significant effect or interaction effect of *OXT* methylation levels for ratings of compassion, therefore this effect was not further explored for interactions with nurturance tendencies.

*3.4.2.2. Reported positive affect.* The effect of *OXT* methylation on reported positive affect in response to the affective images was not further qualified by an interaction with nurturance tendencies, $p = .973$. Lower order effects of affective condition and the interaction between affective condition and *OXT* methylation remained significant, $p < .05$. There was an interaction between affective condition and nurturance tendencies on positive affect, $F(1|154) = 4.98$, $p = .027$. Post-hoc tests revealed that high nurturance tendencies (1 *SD* above the mean) were associated with significantly higher ratings of positive affect in response to positive images compared to low nurturance tendencies (1 *SD* below the mean), $t(154) = 2.97$, $p = .018$, but not in response to negative images, $p = .998$.

### 3.5. Interaction between nurturance tendencies and OXTR methylation levels

#### 3.5.1. EMG analyses

*3.5.1.1. COR activity.* There was a four-way interaction between affective condition, time, *OXTR* methylation levels, and nurturance tendencies, $F(1|1049) = 14.33$, $p < .001$. Lower order effects of affective condition, the interaction between affective condition and time, and the interaction between affective condition, time, and *OXTR* methylation remained significant, $p$'s $< .001$. Post-hoc tests revealed that with *high* nurturance tendencies, COR activity was significantly greater in response to negative images compared to positive images for both low and high *OXT* methylation levels, $-1$ *SD:* $t(1049) = 11.81$, $p < .001$; $+1$ *SD*: $t(1049) = 13.16$, $p < .001$. With *low* nurturance tendencies, COR activity was also significantly greater in response to negative images compared to positive images for both low and high *OXT* methylation levels, $-1$ *SD:* $t(1049) = 11.78$, $p < .001$; $+1$ *SD*: $t(1049) = 3.53$, $p = .010$. Moreover, with *low* nurturance tendencies, low *OXTR* methylation levels were associated with a significantly greater differentiation in COR activity in response to the positive and negative pictures compared to high *OXTR* methylation levels, $t(1049) = 5.12$, $p < .001$. With *high* nurturance tendencies, *OXT* methylation levels were not significantly associated with differences in ZYG activity in response to the affective images, $p = .654$. This suggests that nurturance tendencies buffered the effects of *OXTR* methylation on COR activity in a way that high nurturance tendencies were associated with diminished effects of *OXTR* methylation on COR activity compared to low nurturance tendencies. On the other hand, for low nurturance tendency scores, low *OXTR* methylation was associated with more pronounced COR activity compared to high *OXTR* methylation (see Fig. 5B).

*3.5.1.2. ZYG activity.* There was no significant effect or interaction effect of *OXTR* methylation levels for ZYG activity, therefore this effect was not further explored for interactions with nurturance tendencies.

#### 3.5.2. Behavioral rating analyses

There was no interaction effect of *OXTR* methylation levels for reports of compassion and positive affect, therefore these effects were not further explored for interactions with nurturance tendencies.

## 4. Discussion

The current study aimed to investigate how individual variations in *OXT* and *OXTR* gene methylation levels influenced empathic processes in response to affective images of children. Furthermore, we tested if an interaction with individual variations in care motivation further qualified the association between OXT system gene methylation levels and empathic processes. Our results revealed that overall lower levels of both *OXT* and *OXTR* methylation, which supposedly reflect enhanced OXT system functioning (Haas et al., 2016; Kraaijenvanger et al., 2019), were associated with enhanced empathic processes. Specifically, lower *OXT* methylation was associated with more pronounced COR and ZYG activity, as well as more distinct reports of positive affect in response to the affective images. Lower levels of *OXTR* methylation in turn were related to more pronounced COR activity in response to the affective images. In line with previous studies demonstrating that lower levels of OXT system gene methylation were associated with decreased impairments in social behavior (Haas et al., 2016; Kraaijenvanger et al., 2019), our findings provide further evidence for the suggestion that lower methylation levels in OXT system genes are associated with enhanced social sensitivity. Additionally, our results demonstrated that associations between OXT system gene methylation and empathic processes were partly qualified by an interaction with individual differences in care motivation. Specifically, in participants with low nurturance tendency scores, low *OXT* methylation was associated with more pronounced ZYG activity and low *OXTR* methylation was associated with more pronounced COR activity in response to affective images. Participants with high nurturance tendencies, on the other hand, showed diminished effects of *OXT* and *OXTR* methylation on facial muscle activity. These findings are in agreement with a previous study demonstrating that effects of OXT administration on empathic processes were most pronounced in less socially proficient individuals (Bartz et al., 2010) and substantiates the hypothesis that the OXT system can differentially affect social cognitive processes or prosocial behavior, dependent on individual characteristics (Bartz et al., 2011). Our analyses did not reveal significant interactions between protection tendencies, OXT system gene methylation levels, and empathic processes. There have been studies demonstrating that OXT may promote protective behavior, but such behavior was generally observed towards closely related others, including offspring and in group members (Bos, 2016; De Dreu et al., 2010; Mah et al., 2015). This may partly explain why we did not find such associations in a group of nulliparous women presented with images of unfamiliar children. Moreover, a research effort has determined a critical role for different hormones, most notably testosterone, in relation to protective caregiving behavior (Bos, 2016). Particularly in threatening contexts or when facing infant distress, testosterone may promote protective action (Bos et al., 2021; Van Anders et al., 2012). Therefore, for future research testosterone may be a more likely candidate hormone to examine in interaction with protective tendencies. However, because of the complexity of four-way interactions, these findings should be considered with caution given our sample size.

Importantly, empathy is a multifaceted construct, that involves both bottom-up (or affective) as top-down (or cognitive and regulatory) processes (De Waal and Preston, 2017; Decety and Jackson, 2004). Bottom-up processes of empathy arise when observing or imagining the affective state of another and involve automatic imitation, mimicking, or sharing of the affective states of the other. On the other hand, top-down processes of empathy, influenced by self-regulation and emotional understanding, support the understanding of another's experience and can result in sympathy and compassion, which in turn can facilitate prosocial behavior (De Waal and Preston, 2017; Decety and Jackson, 2004). Our results demonstrated an association between OXT system gene methylation and bottom-up empathic processes, i.e. automatic facial muscle responses and positive affect elicited by affective images. Such bottom-up processes are closely intertwined with top-down processes to evoke the experience of empathy (De Waal and

Preston, 2017), and this is further substantiated by the significant association between an individual's empathic concern and bottom-up empathic processes in our data (see Supplementary data). However, to gain a more complete understanding of how OXT system gene methylation influences empathy, future research would benefit from including experimental measures of cognitive empathy, such as theory of mind and mentalizing tasks.

Our findings contribute towards understanding how the OXT system influences empathy in particular and social behavior and caregiving in general. However, we acknowledge limitations in our study that restrict us from describing a more complete functional model. Firstly, we did not assess concentrations of peripheral OXT in our participants. Initial evidence demonstrated that effects of *OXTR* gene methylation may differ as a function of plasma OXT levels (Ebner et al., 2019), and that *OXTR* gene methylation was related to lower OXT levels (Dadds et al., 2014). However, research on the interplay between peripheral OXT concentrations and OXT system gene methylations is very limited and future research efforts are necessary to provide further knowledge on how they may interact to influence behavioral outcomes. Furthermore, we did not control for genotype variation of participants. Previous studies provide inconsistent results on whether OXT system gene methylation may differ as a function of genotype variation (see, e.g., Chen et al., 2019; Rijlaarsdam et al., 2017; Smearman et al., 2016), yet interactions between methylation and genotype have been observed to predict phenotypic outcomes (see, e.g., Bell et al., 2015; Rijlaarsdam et al., 2017). Integration of both genotypic and epigenetic measures in studies with larger sample sizes would aid future research to gain a greater understanding of the complex interplay between genetic predispositions and epigenetic mechanisms that alter gene activity, which can be modified by environmental exposures (Kraaijenvanger et al., 2019). Additionally, it is important to discuss that we used saliva samples for our DNA methylation analysis. Typically, only a fraction of genes are expressed in a cell, and different cell types emerge through the differential expression of a set of genes (Lodish et al., 2007). Given the cellular heterogeneity of peripheral tissues, such as blood and saliva, this could influence *OXT* gene methylation patterns (Kraaijenvanger et al., 2019). While saliva is heterogeneous, two important studies have demonstrated that overall DNA methylation patterns can provide useful proxies of brain tissue (Braun et al., 2019; Smith et al., 2015). Furthermore, it is demonstrated that *OXTR* methylation patterns correlate significantly in saliva and blood samples (Krol et al., 2019; Puglia et al., 2020), and *OXTR* methylation in blood samples was related with gene expression in brain tissue in both humans and prairie voles (Gregory et al., 2009; Perkeybile et al., 2019). Therefore, saliva samples may be a good proxy tissue when examining behavioral effects that likely derive from differences in the epigenetic programming of brain tissue. Future research, however, may benefit from increased knowledge about how cellular heterogeneity in samples can best be addressed (see, e.g., Zheng et al., 2018). Finally, since our study was part of a series of studies on caregiving behavior prior to motherhood, only nulliparous females were included in our study. Future interdisciplinary studies are necessary to demonstrate how basic empathic processes are related to actual sensitive behavior when interacting with children, and to test if sex differences may arise in the association with OXT system gene methylation.

Despite its limitations, the current study demonstrates that epigenetic control of OXT system genes is associated with empathic processes in response to children. Overall, lower methylation levels in the *OXT* and *OXTR* genes, which supposedly reflect an enhanced functioning of the OXT system, were related to enhanced bottom-up empathic processes. Furthermore, the association between OXT system gene methylation and empathic processes in response to children was partly qualified by an interaction with individual nurturance tendencies. Since empathy is an essential part of sensitive caregiving (De Waal and Preston, 2017; Decety and Jackson, 2004), these findings provide further insight into possible biological mechanisms underlying maladaptive caregiving behavior, which will ultimately result in more efficient approaches to reduce the prevalence of such behavior and minimize the impact on affected children (Bos, 2016).

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.psyneuen.2021.105629.

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